Use of a small-scale, portable test chamber for determining the bactericidal efficacy of aerosolized glycol formulations

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Significance and Impact of the study: There is an increased interest in developing effective microbicidal aerosolised formulations. The development of a small in-house test chamber allowed the measurement of the microbicidal efficacy of an aerosolised glycol/ethanol formulation at a low cost. We showed that a glycol/ethanol aerosolised formulation caused extensive structural damage in Gram-negative and -positive bacteria resulting in a 3 log_{10} reduction in viability.

Abstract

This study aimed to understand the efficacy and mechanisms of action of an aerosolised glycol-ethanol formulations against bacteria. We validated a small scale in-house test chamber to determine the microbicidal efficacy of four aerosolized formulations combining dipropylene glycol and ethanol against *Staphylococcus aureus* and *Escherichia coli* embedded in alginate. The aerosolised glycol/ethanol formulation decreased bacterial viability by 3 Log_{10} and was more efficacious than an ethanol only control formulation. Electron microscopic examination indicated extensive structural damage in both bacteria, and membrane damage was confirmed with potassium release in *S. aureus* and DNA release in *E. coli*. The development of a small test chamber facilitated the measurement of the microbicidal efficacy and experiments to understand the mechanism of action of an aerosolised microbicidal formulation.

Introduction

Pathogens discharged into the air may settle on environmental surfaces, which could then become secondary vehicles for the spread of infectious agents indoors occurring at the air-surface-air nexus (Gralton *et al*., 2011; Ijaz *et al*., 2016). Considering the concern about the potential spread of microorganisms indoors and the limited techniques for testing new formulations, Sattar and colleagues (2016) designed a protocol to study survival and inactivation of human pathogens in indoor air. These authors tested the efficacy of a microbicidal formulation containing 4% dipropylene glycol (DPG) and 35% ethanol distributed within a 24.3 m^3 aerobiology chamber using an air purifier with a newly-designed fogger (Sattar et al. unpublished data).

The study of human pathogens aerosols requires the ability to produce them experimentally in appropriate droplet size and sample them safely for analysis over a predetermined time periods (Sattar and Ijaz, 1987). Since the recovery of aerosolised bacterial inocula from large-scale microbicide experiments presents several technical challenges, the immobilization of bacteria in an alginate support may provide an appropriate alternative (Shackelford *et al.,* 2006). The alginate matrix provides sufficiently large pores to allow the easy penetration of microbicide and may enable testing of microbicidal efficacy without the confounding effects of bacterial aerosol-induced cell injury or drying (Tattawasart *et al*., 2000a; Shackelford *et al*., 2006) or aerosolising bacteria with the negative effects of aerosolising process on bacterial viability (O'Jeil *et al.,* 2013). In addition, the use of an alginate matrix allows recovering bacteria in high number for the study of mechanisms of action (Tattawasart *et al*., 2000a; Shackelford *et al*., 2006). Glycols such as propylene glycol (PG) and triethylene

glycol (TEG) are greatly used in formulations, generally as solvent, extractant and on occasions as preservatives at concentrations 15-30% (Rowe *et al.,* 2009). The microbicidal activity of glycols has however not been widely reported.

The aim of this study was to measure the bactericidal efficacy of several aerosolised glycol-based formulations and their mechanisms of microbicidal action using a small scale aerosolisation test chamber.

Results and discussion

There is a great interest in gaseous and air decontamination technologies notably in healthcare settings (Davies et al., 2011). The use of highly reactive chemistry such as vaporized hydrogen peroxide has been successful in controlling pathogen outbreaks in healthcare environments (Ray et al., 2010; Goyal et al., 2014). Aerosolised chlorine-based microbicides have also been explored against *Staphylococcus aureus* with various degrees of efficacy (Thorn et al., 2015). However, these highly reactive chemistries are toxic and the room to be decontaminated needs to be vacated and sealed during the gaseous process. In addition, testing the efficacy of aerosolised formulations is expensive and require the use of specifically designed aerobiology test chambers (Sattar et al., 2016). Here, we successfully developed a small scale test chamber to study the efficacy and mechanisms of action of aerosolised formulations. The combination of the chamber with an immobilised bacterial inoculum allowed the recovery of a high bacterial inoculum facilitating the study of the formulations' mechanisms of action.

97

98 *Effect of liquid formulations on bacterial growth*

99 We are not aware of any scientific publications describing of the microbicidal
100 activity of dipropylene glycol. Our results show that the test formulations,
101 including dipropylene glycol only (6.67%) inhibited the growth of the test
102 bacteria. All liquid formulations affected bacterial growth in comparison to the
103 TSB positive growth control (Figures 2 and 3). The formulations containing
104 glycol, or ethanol or a combination of glycol and ethanol had a more
105 pronounced effect inhibiting completely the growth of both bacteria comparing
106 to the blank formulation that affected bacterial growth rate and final OD value
107 (Figures 2 and 3).

108

109 *Bactericidal efficacy of aerosolised formulations*

110 Percentage recoveries of bacteria embedded in the alginate were 90.8% and
111 91.63% for *E. coli* and *S. aureus*, respectively. The use of the fogger
112 formulations containing glycol in our test chamber (10 sec aerosol exposure,
113 40-60% RH) produced a reduction in bacterial viability. However, the complete
114 formulation (Glycol + Ethanol) was significantly (ANOVA; $P<0.001$) more
115 efficacious with a reduction of 3.20 ± 0.13 Log_{10} for *S. aureus aureus* and 3.19
116 ± 0.39 Log_{10} cfu ml^{-1} for *E. coli* compared to 1.54 ± 0.31 and 1.41 ± 0.15 with
117 each bacterium respectively (Table 3). There were no statistically significant
118 differences (ANOVA; $P>0.1$) between the efficacy of the glycol only, the ethanol
119 only formulations and the blank formulation (Table 3). The use of chlorine-
120 based aerosols (with 20 min aerosol exposure, 10 min resting time, and 50%RH)
121 produced 1-5 log_{10} reduction in *S. aureus* concentration on stainless steel

surfaces depending on the initial chlorine solution (sodium hypochlorite, chlorine dioxide or electrochemically activated solution) (Thorn et al., 2013). Gaseous chlorine (250 mg l^{-1}) has been used *in situ* to decontaminate indoor air pathogens and contributed to a reduction <1500 cfu m^3 in bacteria and of <1000 cfu m^3 in fungi (Hsu et al., 2015).

The bactericidal activity of other glycols has been reported. Berry (1944) reported MIC ethylene glycol monophenyl ether of 0.8% v/v against *S. aureus* and 0.5 % v/v against *E. coli* while a number of chloro-and methyl-substituted aryl ethers of glycerol, propylene glycol and trimethylene glycol were shown to have some activity against bacteria, fungi and yeast (Berger et al., 1953). Propylene glycol has been used as a solvent in combination with phenol with report of some bactericidal activity (Baker and Twort, 1941). It is however clear that RH played an important role in the activity of the formulation (Baker and Twort, 1941). Chirife and colleagues (1983) studied the microbicidal activity of polyethylene glycol 400 and suggested that the bactericidal activity observed at 35°C was a combination of lowering water activity and a direct effect of bacterial cells demonstrated by cell clumping. Recently ethylene glycol bactericidal activity was reported against *E. coli* with MIC and MBC values of 18 and 24 % v/v (Moghayedi et al. 2017).

Mechanisms of bactericidal action of aerosolised glycol formulations

The mechanisms of action of the aerosolised formulations were explored. It was hypothesized that the bactericidal activity observed resulted from membrane damage. Using the FEI Quanta 200F software (Eindhoven, The Netherlands), direct SEM examination showed evidence of structural damage for both *S.*

147 *aureus* and *E. coli* with all the formulations tested (Figures 4 and 5). Ninety
148 percent of the bacterial population was damaged following exposure to the
149 control formulations (Table 3). Such level of inactivation contributes to create a
150 substantial artefact as demonstrated from the SEM images and OMP analysis.
151 Loss of bacterial viability during sample preparation prior to testing has been
152 well reported. The decrease in bacterial concentration depends on the material
153 used (Best et al., 1988; Thorn et al., 2013) and the type of bacteria; some recent
154 European efficacy test protocol such as the EN14776 recommends the use of
155 glycerol with the Gram-negative bacterial inoculum to protect test inocula from
156 dehydration. Here, the use of the alginate limited bacterial loss and contributed
157 to the use of a highly reproducible test inoculum concentration, as described by
158 Shackelford and colleagues (2006).
159 It is clear that the aerosol of the blank formulation by itself caused membrane
160 damage, with indication that the Gram-negative was more affected with release
161 of DNA, although not to the OMP. Although the extent of damage could not be
162 quantified, the combined formulations seems to disrupt structural integrity of *S.*
163 *aureus* more extensively when compared to the other formulations (Figure 4).
164 The severity of damage caused by the combined formulation was more
165 apparent in *E. coli* (Figure 5). Exposure to the glycol only or ethanol only
166 formulation seems to 'smooth' the surface of *E. coli* while the blank formulation
167 affected the bacterial surface but not to same extent as the combined
168 formulation (Figure 5). SEM images indicated structural changes when bacteria
169 were exposed to the formulations with the different formulations. Damage
170 caused by the combined glycol and ethanol formulation seemed more severe
171 in both Gram-negative and Gram-positive bacteria. A recent study showed

172 severe bacterial structural damage in *E. coli* exposed to 25% ethylene glycol
173 (Moghayedi et al. 2017).
174 Potassium leakage measurements indicated that the full formulation may
175 interact with the cytoplasmic membrane of the Gram-positive bacterium,
176 releasing some potassium, but not larger cell components. Potassium release
177 is the first indicator of membrane damage followed by larger cytoplasmic
178 constituents (Maillard, 2002). Here, a small but statistically significant increase
179 (ANOVA; $P< 0.001$) in potassium leakage was observed when testing the glycol
180 and ethanol (A), and glycol only formulations (B) against *S. aureus* compared
181 to the blank formulation (D) (Table 4). No potassium was released from *E. coli*
182 exposed to the aerosolised formulations (Table 4).
183 The release of DNA from bacteria following exposure to the aerosolised
184 formulations was measured spectrophotometrically following exposure. The
185 OD_{260} reading allows for the calculation of the concentration of nucleic acid in
186 the sample. An OD of 1 corresponds to ~50 μg ml^{-1} for DNA. The OD_{260}/OD_{280}
187 ratio reading provides an estimate of the purity of the nucleic acid. Pure
188 preparations of DNA have OD_{260}/OD_{280} values of ≥1.8. There were more DNA
189 in the *E. coli* samples than in the *S. aureus* ones (Table 5). Exposure to the
190 aerosolised formulation contained glycols and ethanol or to the nebulised blank
191 formulation resulted in very little DNA release, 4% and 6% of total estimated
192 DNA in *S. aureus* and *E. coli* respectively.
193
194 The results of SDS-PAGE indicated no changes in OMP in *E. coli* after
195 exposure to the blank and the formulation containing combined glycols and
196 ethanol were detected by the technique. There was no apparent change in the

OMP profile following exposure to the blank or the combined glycol and ethanol formulation, although an increase in the band concentration of three specific OMP of 35 kD, 27 kD and 16 kD approximately was observed when bacteria were exposed to the aerosolised formulations (Figure 6).

In conclusion, the test chamber described in our study allowed the microbicidal evaluation of foggers containing glycol-based formulations and also nebulization of blank formulation against bacteria embedded in alginate matrix. The advantage of the small size chamber is the rapidity of the experiment and cost to set up (compared to the use of an aerobiology chamber). One limitation is that the aerosolised formulations need to be adapted to the small volume of our test chamber. The bactericidal efficacy of the combined aerosolised glycol and ethanol formulation could be partly attributed to damaging the cytoplasmic membrane of Gram-positive bacteria and to damaging the outer membrane of Gram-negative bacteria.

Material and methods

Aerosolized Formulations

Four formulations (Table 1) combining DPG and anhydrous ethanol provided by Reckitt Benckiser (One Philips Parkway, Montvale, NJ 07645. USA) were studied. Samples were stored at room temperature prior to fogging.

Bacterial strains

Staphylococcus aureus (ATCC 6538) and *Escherichia coli* (ATCC 11229) commonly used in standard efficacy test protocols were used as test bacteria.

Both strains were stored on Nutriprotect beads (Fisher Scientific, Loughborough, UK) at −80 ± 1°C and restricted to a maximum of 2 subcultures from the original freezer stock prior to any testing. Both strains were grown in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) at 37 ± 1°C for 16-24 hours. Test inoculum were prepared from harvesting an overnight TSB culture centrifuged at 5,000 g for 10 minutes and re-suspended in phosphate buffer saline (PBS) (Fisher Scientific, Loughborough, UK).

Sodium alginate test inoculum preparation

The effects of aerosolised formulations were analysed against vegetative bacteria embedded in sodium alginate. The test protocol was adapted from the procedure described in Shackelford *et al*. (2006). A 1 ml sample of bacterial suspension was added to a 1 ml cooled 3% (w/v) sodium alginate solution in deionised water and mixed by pipetting (BDH Chemicals Ltd, Poole, UK). The final concentration in the bacteria/alginate mixture was 1–10 x 10^9 cfu ml^{-1}. Aliquot of alginate/bacteria (0.2 ml) was dispensed onto the centre of a moulds holder system of M8 flat stainless still washers placed on top of stainless still coupons grade 2B of 0.22 mm X 0.22 mm (Goodfellows Cambridge Ltd. Huntington, UK). Both, flat washers and coupons were soaked with 5 % Decon90 (Decon Laboratories Limited, Hove, UK) in deionised water for 60 min, rinsed, dried and then autoclaved before use.

Loaded mould holder system was placed into individual wells of sterile 6 well plates (Corning® Costar®, Sigma-Aldrich UK) containing 10 ml of 2% calcium chloride solution (BDH chemicals, Poole, UK) and left for 5 min to form a gel as

246 the sodium ions are exchanged with calcium ions and the polymers become
247 cross-linked (Waldman *et al*., 1998).
248 To recover the bacteria from the alginate matrix, both control (no formulation
249 added) and post formulations exposure, the gels were rinsed in five changes of
250 10 ml of sterile distilled water and dissolved in 10 ml of McIlvaine's buffer (0.1
251 mol l^{-1} citric acid and 0.2 mol l^{-1} disodium phosphate at pH 7.4; Fisher Scientific).
252 Samples were taken from the dissolved gels, serially diluted in sterile PBS (pH
253 7.4; Fisher Scientific) and CFU counts were performed using the Miles and
254 Misra drop count method. The bacterial recovery and microbicidal efficacy (BE)
255 was calculated as follows: BE= log N_c – log N_b where N_c and N_b represent the
256 numbers of CFU ml^{-1} in the control and biocide fogger formulations, respectively.
257
258 *In-house test chamber conditions*
259 A small portable aerosolisation test chamber was modified from the test
260 chamber described by O'Jeil and colleagues (2013). Fogger release conditions
261 were adapted to the size of the chamber consisted of a 23 cm stainless steel
262 tube connected at the other end to an Andersen cascade impactor (Westech
263 Instrument Services Ltd, Henlow, UK). A constant low flow rate measured by a
264 Copley Scientific DFM2000 (Nottingham, UK) flow meter was generated
265 through a vacuum pump (Fisherbrand, Loughborough, UK) connected to the
266 Andersen cascade impactor (Figure 1). Temperature and relative humidity
267 conditions on surfaces were determined using a S154TH temperature and
268 relative humidity probe. Testing parameters were established according to the
269 chamber dimension size. Test operation conditions (Table 2) were set up to
270 mimic a previous study performed with the aerobiology chamber (Sattar et al.,

271 2016). The procedure was carried out in a class-2 microbiological safety
272 cabinet facility.
273 The blank formulation (Table 1) could not be delivered by fogger due to
274 excessive foam release. Instead the blank formulation was nebulised using a
275 nebuliser (Philips Respronics, Best, The Netherlands) connected to the 23 cm
276 stainless steel tube of the rig. All test parameters were the same for all tested
277 formulations.
278
279 *Bactericidal activity of formulations in suspension*
280 Bacterial growth kinetics was determined using the Bioscreen C Microbial
281 Analyser (Labsystems, Helsinki, Finland) for both microorganisms using the
282 four formulations as solutions: glycol+ethanol (A), glycol (B), ethanol (C) and
283 blank (D). Controls consisted of each bacterium growing in TSB. The Bioscreen
284 was run for 24 h at 25°C and readings were taken using a wideband filter (420-
285 580nm) every 15 min preceded by 10 s shaking.
286
287 *Microbicide mechanisms of action*
288 Bacterial gross structural damage following exposure to aerosolised
289 formulations was explored by scanning electron microscopic examination
290 (SEM imaging) (Walkera *et al*., 2003) using the FEI Quanta 200F (Eindhoven,
291 The Netherlands). Loss of (cytoplasmic) membrane integrity was measured
292 with potassium leakage according to Walsh *et al*. (2003). A number of controls
293 were performed including boiled bacteria at 80ºC for 20 min (maximum
294 potassium release; positive control 1), boiled bacteria embedded in the matrix
295 at 80ºC for 20 min (effect of alginate on potassium release; positive control 2),

296 effect of nebulisation on the release of K^+ from embedded bacteria in the
297 alginate (nebulisation control). Potassium release was measured by inductively
298 coupled plasma mass spectrometry (Agilent 7900 ICP-MS). A five-point
299 calibration (1, 0.1, 0.01 and 0.001 mg l^{-1}, (Tune) and calibration blank (CAL))
300 was run and an internal standard (IS) was used throughout the analysis. All
301 standards (Tune/Calibration and IS) are certified reference standards from
302 Agilent. Samples were run in duplicate with blanks in between different samples
303 to ensure there was no carryover of K^+.
304 To evidence gross membrane damage, DNA release was measured by UV
305 spectrometry following exposure to the four formulations. Following exposure
306 to aerosolised formulation and dissolution of the alginate, samples were added
307 to a 1 cm path-length cuvette and OD recorded 260 nm and at 280 nm. Samples
308 with an OD of 1 at 260 nm contain approximately 50 µg ml^{-1} double-stranded
309 DNA (Nicklas and Buel, 2003).
310 Positive controls consisted in DNA measured spectrophotometrically of boiled
311 bacteria at 80ºC during 20 min.
312 Damage to the Gram-negative outer membrane proteins (OMP) after exposure
313 to the different formulations were examined using SDS-PAGE according to
314 Tattawasart and colleagues (2000b) with the silver staining performed
315 according to Hitchcock and Brown (1983). ImageJ software (Schindelin *et al.*,
316 2012) was used as a semi-quantitative tool to determine the amount of OMP
317 observed.
318
319 *Statistical analysis*

All experiments were performed in triplicate unless otherwise stated. Bacterial reduction and effects of the possible mechanism of action of the formulations tested were compared by one-way ANOVA and Tukey tests with a 95% confidence level using Rstudio software (Version 1.1.383). Results were considered significant when $P < 0.001$.

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Conflict of interest

JR Rubino and Ijaz MK are employees of Reckitt Benckiser.

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417
418

419

420

421 **Table 1** Aerosolized formulations tested

Sample	Active	Alcohol	Concentration (%)
Full formulation (A)	Dipropylene glycol	Anhydrous ethanol	6.67% / 46.67%
Glycol formulation (B)	Dipropylene glycol	-	6.67%
Ethanol formulation (C)	-	Anhydrous ethanol	46.67%
Blank formulation (D)	-	-	0

422

423 **Table 2** In-house chamber testing conditions

Parameter	Specification	Condition tested
Chamber dimension	Size	230 cm^3
Test operation	Air rate (flow)	2 l min^{-1}
	Soil load	-
	Fogger release	10 sec
	Relative humidity	40-60%
	Temperature	24°C
Glycol release	Concentration	0.18 g
Microorganisms tested	Bacterial concentration	1 x 10^9 CFU ml^{-1}

424

425 **Table 3** Bactericidal efficacy of aerosolised formulations against *S. aureus* and *E. coli* with a flow of 2.0 l min^{-1}.

Formulation	*S. aureus* (Log_{10} ± SD)			*E. coli* (Log_{10} ± SD)		
	T=0	T=10 sec		T=0	T=10 sec	
		Recovery	Reduction		Recovery	Reduction
Complete formulation- Glycol and Ethanol	9.93 ± 0.15	6.73 ± 0.04	3.20 ± 0.13	9.93 ± 0.05	6.74 ± 0.33	3.19 ± 0.39
Glycol only formulation	9.68 ± 0.00	8.12 ± 0.20	1.56 ± 0.20	9.68 ± 0.11	7.81 ± 0.18	1.87 ± 0.26
Ethanol only formulation	9.68 ± 0.00	7.90 ± 0.20	1.78 ± 0.20	9.68 ± 0.11	7.61 ±0 .44	2.07 ± 0.52
Blank formulation	9.38 ± 0.00	7.84 ± 0.31	1.54 ± 0.31	9.07 ± 0.00	7.66 ± 0.15	1.41 ± 0.15

426

Table 4 Potassium release from *S. aureus* and *E. coli* after exposure with aerosolised or nebulised formulations.

SAMPLE (n=2)	Potassium Release (ppm) *S. aureus*	*E. coli*
Positive control: bacteria only	100.99	26.75
Positive control: bacteria + matrix	48.51	12.36
Nebulisation control	0.57	0.14
Blank formulation	0.69	0.19
Complete formulation-Glycol and Ethanol	0.91	0.26
Glycol only formulation	0.87	0.19
Ethanol only formulation	0.49	0.18

Table 5 DNA release from *S. aureus* and *E. coli* following exposure to the aerosolised formulations.

SAMPLE (n=2)	OD_{260}	OD_{280}	OD_{260}:OD_{280}	Estimated DNA concentration ($\mu g\ ml^{-1}$)
S. aureus				
Positive control (boiled bacteria)	0.50	0.20	2.5	25.0
Complete formulation-Glycol and Ethanol	0.02	0.02	1.00	1.0
Blank formulation	0.02	0.01	1.67	1.0
E. coli				
Positive control (boiled bacteria)	3.00	2.02	1.49	150
Complete formulation-Glycol and Ethanol	0.19	0.09	2.01	9.5
Blank formulation	0.13	0.07	1.87	6.5

Figure 1. In-house aerosolised test chamber

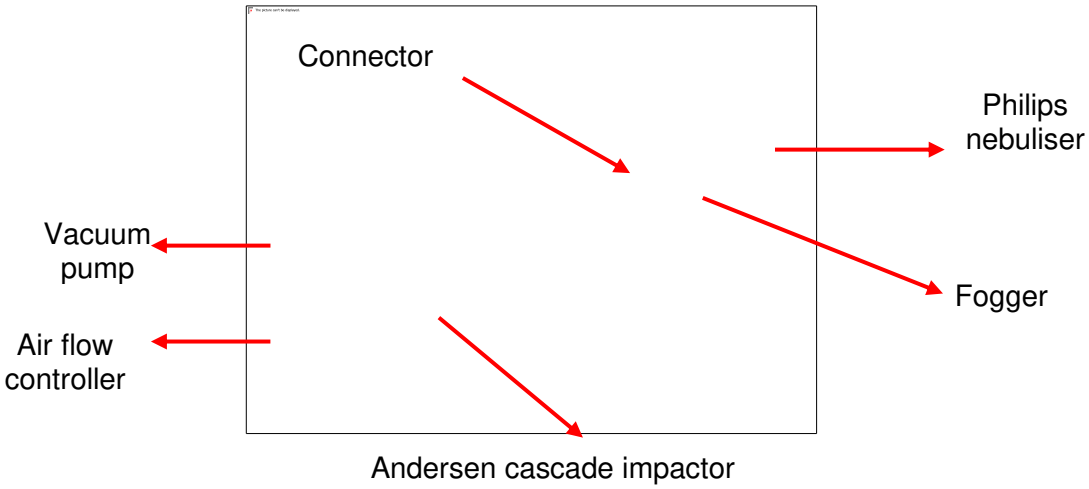


Figure 2 Effect of the tested formulations on the growth of *S. aureus.* (■) Complete formulation-Glycol and Ethanol; (■): Glycol only formulation; (■) Ethanol only formulation; (■): Blank formulation and (■): growth control (TSB)

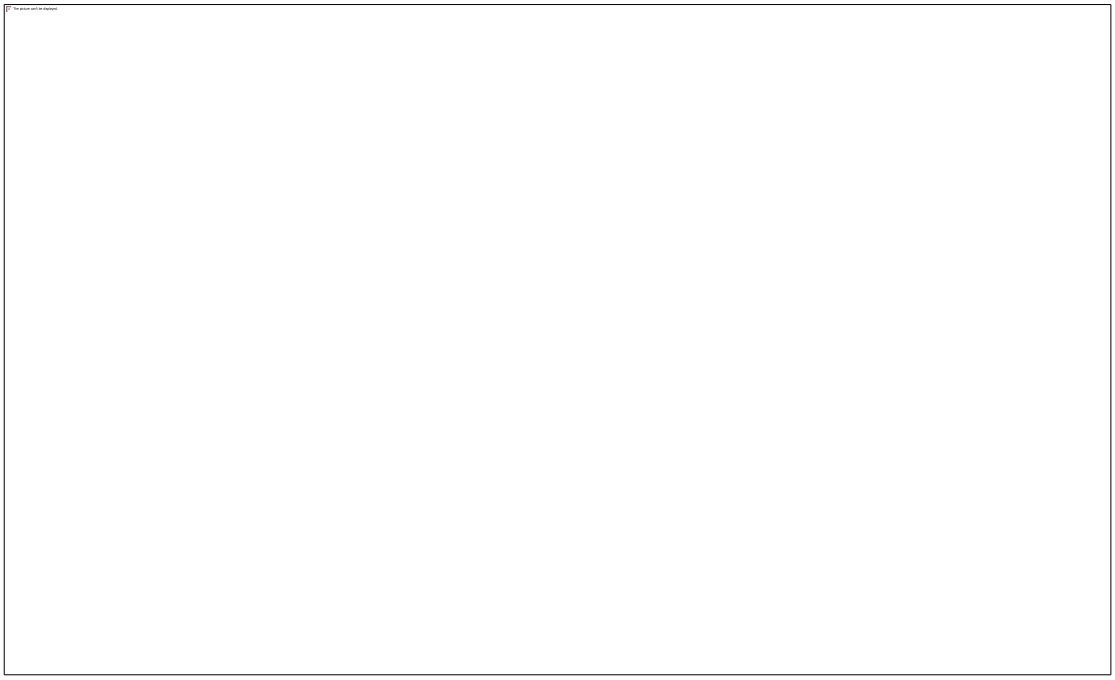


Figure 3 Effect of the tested formulations on the growth of *E. coli.* (■) Complete formulation-Glycol and Ethanol; (■): Glycol only formulation; (■) Ethanol only formulation; (■): Blank formulation and (■): growth control (TSB). (n=32)

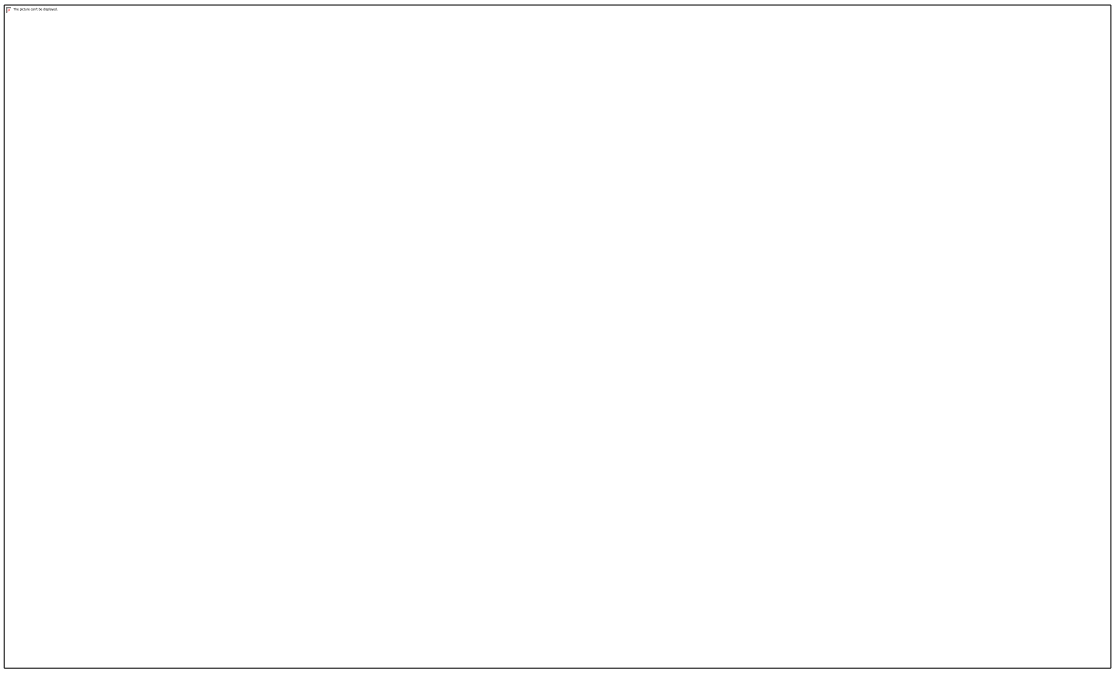
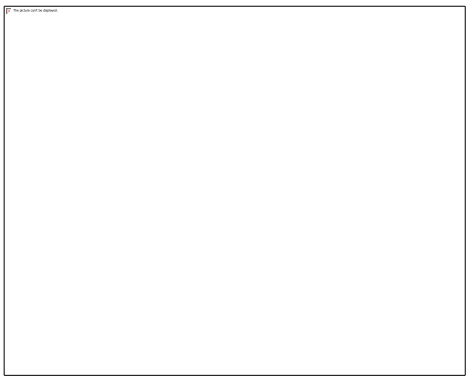
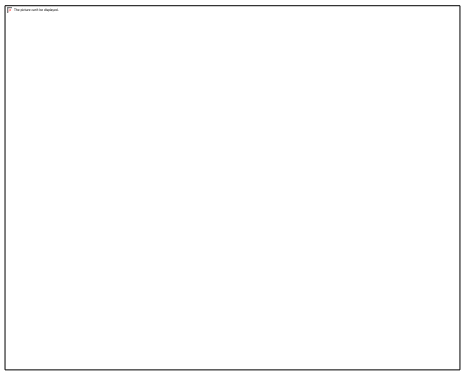


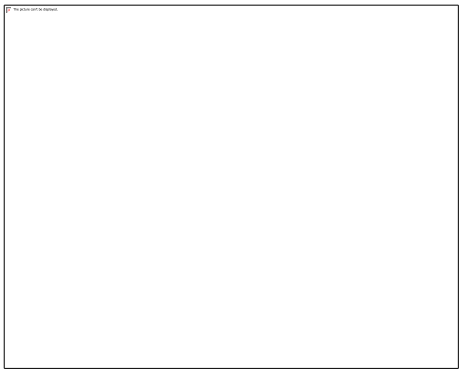
Figure 4 SEM images of *S. aureus* after exposure to aerosolised formulations. (A) Complete formulation-Glycol and Ethanol; (B): Glycol only formulation; (C) Ethanol only formulation; (D): Blank formulation. Bacteria were coloured using the GNU Image Manipulation Program (GIMP) version 2.8.22.

(A) (B)





(C) (D)



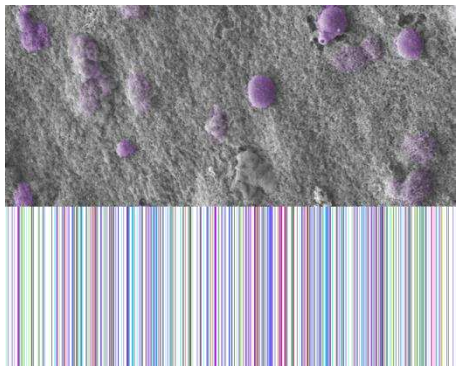
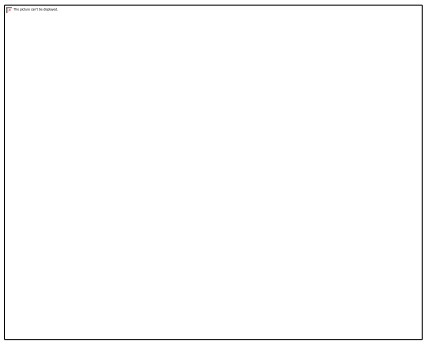
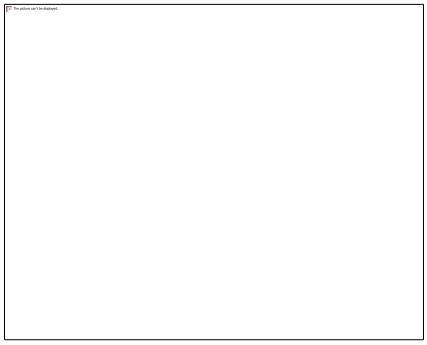


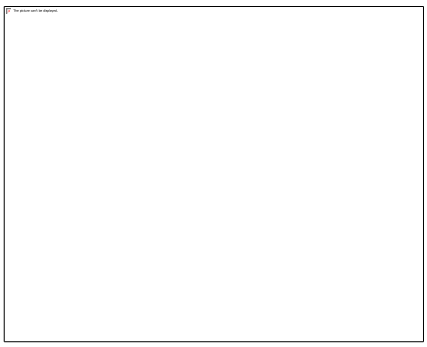
Figure 5 SEM images of *E. coli* after exposure to aerosolised formulations. (A) Complete formulation-Glycol and Ethanol; (B): Glycol only formulation; (C) Ethanol only formulation; (D): Blank formulation. Bacteria were coloured using the GNU Image Manipulation Program (GIMP) version 2.8.22.

(A) (B)





(C) (D)



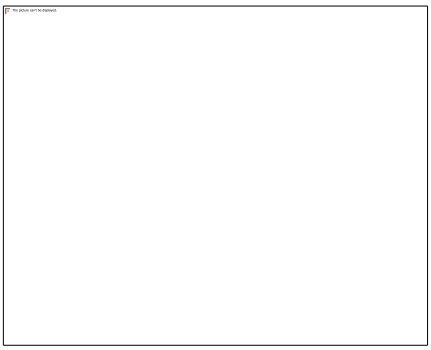


Figure 6 SDS-PAGE analysis of outer membrane proteins. Line 1: Standard molecular weight; line 2: control *E. coli* without aerosolized formulation; line 3. *E. coli* treated with the blank formulation (D) and line 4: *E. coli* treated with the glycol and ethanol formulation (A).